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CELL DIVISION INHIBITION OF ARBACIA AND CHAETOPTERUS EGGS AND ITS REVERSAL BY KREBS CYCLE INTERMEDIATES AND CERTAIN PHOSPHATE COMPOUNDS [1, 2]

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It has been demonstrated that anoxia (Loeb, 1895), cyanide (Lyon, 1902), azide (Clowes and Krahl, 1939), and dinitrophenol (DNP) (Clowes and Krahl, 1936) inhibit the cleavage of *Arbacia punctulata* eggs. These metabolic inhibitors are known to interfere with hydrogen transport (Stannard and Horecker, 1948) and/or phosphorylation (Reiner and Spiegelman, 1947). Because "high energy phosphate" ($\backsim$P) is equilibrated with the adenosine triphosphate (ATP) within the cell (Green and Colowick, 1944), it was considered possible that the inhibition of cleavage, produced by the above agents, might be reversed by addition of ATP.

Further, because current ideas relate the oxidative formation of $\backsim$P to the functioning Krebs cycle (Lipman, 1941), inhibition of the cycle by malonate might inhibit cleavage because of $\backsim$P lack. If the general scheme of $\backsim$P generation applied to *Arbacia,* relief of the malonate division inhibition would be effected by succinate, a substance which competes with malonate for succinic dehydrogenase; fumarate, an intermediate of the Krebs cycle beyond the point of action of malonate (Potter and Dubois, 1943); and ATP, the postulated critical product of the cycle (Lipman, 1946).

Two reasons prompted an extension of the experiments to include *Chaetopterus,* as well as *Arbacia,* eggs: (1) *Arbacia* eggs show an increase, and *Chaetopterus* eggs a decrease, in oxygen consumption at the time of initiation of cell division (Whitaker, 1932); (2) if *Arbacia,* an Echinoderm, and *Chactopterus,* an Annelid, exhibit similar reactions to malonate, it could indicate a general importance of the Krebs cycle and ATP to cell division in aerobic organisms.

[1] The author would like to express his sincere gratitude to Dr. J. O. Hutchens, Dr. D. L. Harris and Dr. J. Z. Hearon for their encouragement and constructive criticism. Dr. G. H. A. Clowes was kind enough to make the Eli Lilly Laboratories at Woods Hole available for part of this work. Grateful acknowledgment is made to Manfred Brust for technical assistance.

[2] Completed in part while Fellow of the American Cancer Society (recommended by the Committee on Growth of the National Research Council).

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Methods

Arbacia punctulata and *Chaetopterus pergamentaceus* eggs were washed in sea water and adjusted to 0.1 to 0.2 per cent by volume by taking a small aliquot of the egg suspension and centrifuging it at 1000 times gravity in duplicate hematocrit tubes. From the egg volume the number of eggs present in each experimental vessel was calculated (Harvey, 1932; Krahl, 1950). A second aliquot of eggs was fertilized and the per cent cleavage noted at the end of one hour as a test of both egg and sperm viability before use. Egg suspensions were used only if they showed 90 to 100 per cent cleavage. The fertilized eggs were added to the experimental vessels after 25 minutes (*Arbacia*) or 40 minutes (*Chaetopterus*).

In low oxygen tension experiments, mixtures of air and nitrogen were prepared by displacement of water in a calibrated glass bottle (Umbreit *et al.*, 1949). The bottle was connected by rubber tubing to serially arranged double-arm Warburg vessels which contained, in the main compartment, fertilized eggs and reversing agent or NaCl. The rate of gassing was maintained approximately constant by regulating the rate of bubble evolution by means of a screw clamp placed at the end of the last tube which dipped below the surface of the water bath. The gas mixture was passed through the serially connected vessels for 30 minutes with shaking at an amplitude of six centimeters and rate about 120 per minute. At the end of the gassing period the vessels were closed and the shaking continued for an additional two hours. The control differed from the experimental vessels only in the substitution of air for the gas mixture. The disadvantage of a serially connected system is the possibility of unequal gassing of the individual flasks. To minimize this disadvantage, the order of the flasks was varied from experiment to experiment. To insure adequate removal of air, the volume of flushing gas used was 20 times the volume of the empty system.

When azide, cyanide, and DNP [4] were used as division-inhibiting agents, the experiments were conducted in air-filled closed vessels to which 3.3 mg. per ml. of glycylglycine buffer was added at pH 8.2. No buffer was required with malonate and its reversing agents, succinate and fumarate, because the latter solutions were adjusted to pH 8.2 with solid sodium hydroxide. Two types of experiments were performed when malonate was used to inhibit cell division. In some experiments solid sodium hydroxide was added to malonic acid dissolved in sea water which produced a hypertonic solution. In other experiments solid sodium hydroxide was added to malonic, fumaric or succinic acid dissolved in distilled water to produce a solution isotonic with sea water. Experimental temperature varied between 22–27° C., but within a single experiment the temperature was constant to ± 0.1° C.

In all instances at the conclusion of the experiment (two to three hours) egg cleavage was stopped with formaldehyde and the number of divisions per egg in each vessel was calculated according to the method of Smith and Clowes (1924). In this calculation 100 eggs are counted and the cleavage stage of each egg is recorded as follows: no divisions, one cell; one division, two cell; two divisions, four cell; etc. The sum of the number of divisions divided by 100 yields a number designated as the number of divisions per egg.

[4] The DNP was a purified sample obtained through the kindness of Dr. G. H. A. Clowes of the Eli Lilly Laboratories, Woods Hole, Mass.

Experimental Results

Interference with the hydrogen transport system near its terminus

Graphically presented in Figure 1 are the results of typical experiments in which low oxygen tension was used to inhibit cleavage of *Arbacia* eggs. It is seen that in all instances ATP stimulated cell division of the inhibited eggs. Further, the added ATP completely reversed the division inhibition provided the latter was not greater than 50 per cent. Figure 2 shows that adenylic acid (AA),

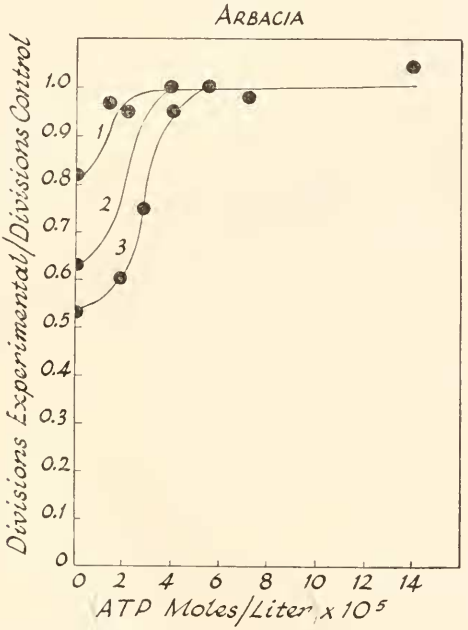

FIGURE 1. The reversal effect of ATP on low oxygen tension-inhibited *Arbacia* eggs. Rate of oxygen consumption as per cent of control: (1) 75; (2) 60; (3) 50. Experimental duration 100 minutes.

hydrolyzed ATP (HATP), or the ~ P compound, inorganic triphosphate (ITP), were not effective in reversing the division inhibition.

Because added ATP completely reversed low oxygen tension inhibition of *Arbacia* egg cleavage, it was thought a similar reversal might be obtained when cyanide or azide were used as inhibiting agents. The results with sodium azide are presented in Figure 3. The results with sodium cyanide are presented in Figure 4. It is seen that the inhibition of cleavage in *Arbacia* eggs is:

(1) Reversed when ATP is added to cyanide-inhibited cells.
(2) Not reversed when ATP is added to azide-inhibited cells.
(3) Not reversed when hydrolyzed ATP is added to either cyanide- or azide-inhibited cells.

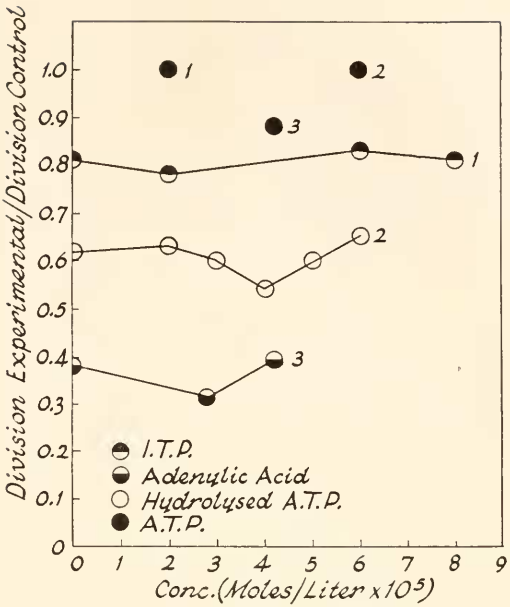

FIGURE 2. The effect of ATP, ITP, AA and HATP on low oxygen tension—inhibited *Arbacia* eggs. The corresponding numbers are the results obtained with the same batch of eggs. Experimental period 100 minutes.

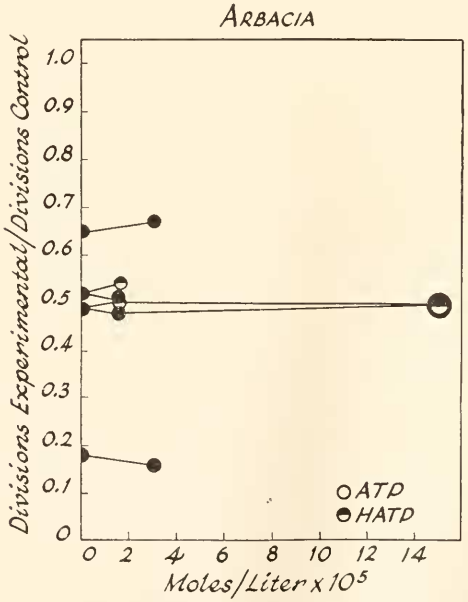

FIGURE 3. The effect of ATP and HATP on azide-inhibited *Arbacia* eggs. With increasing inhibition the concentration of NaN_3 was: 1.35, 1.25, 2.7×10^{-3} *M* sodium azide, and the experimental time was 180, 100 and 180 minutes, respectively.

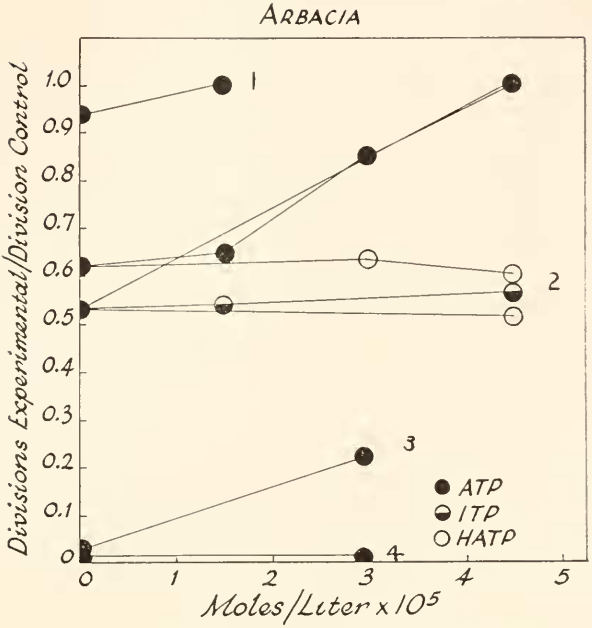

FIGURE 4. ATP, ITP and HATP effect on cyanide-inhibited *Arbacia* eggs. Cyanide concentration (1) 2; (2) 6; (3) 8; (4) 16×10^{-5} *M*. Experimental time 100 minutes.

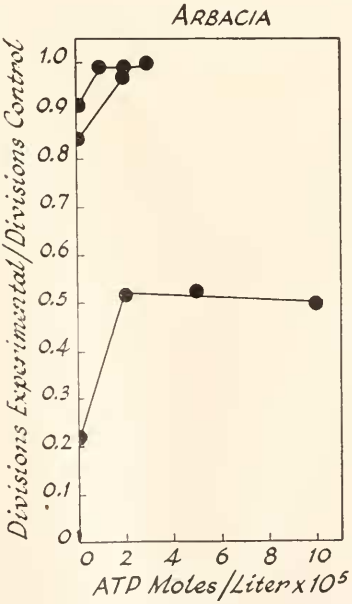

FIGURE 5. ATP effect on DNP-inhibited *Arbacia* eggs. Concentration of DNP with increasing amount of inhibition: 0.4, 0.8 and 1.6×10^{-5} *M*. Experimental time 100 minutes.

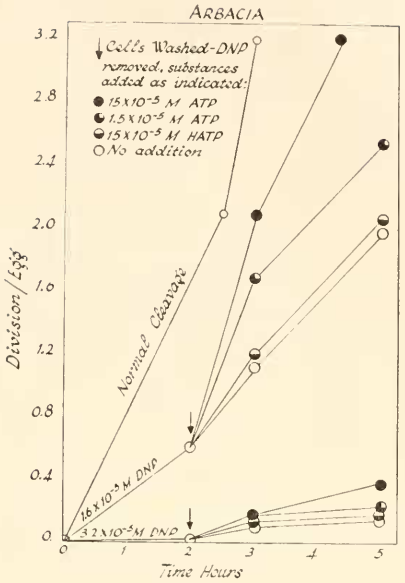

FIGURE 6. Recovery of *Arbacia* eggs following DNP inhibition.

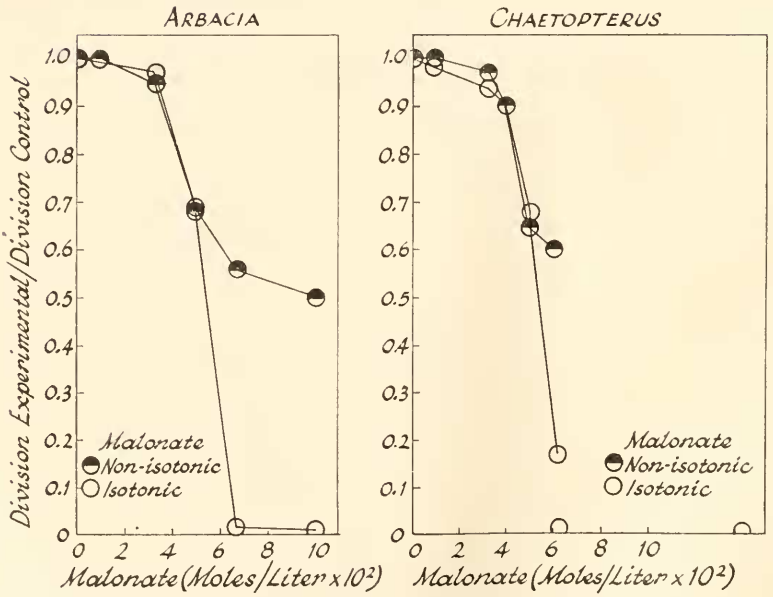

FIGURE 7. The inhibitory effect of isotonic and non-isotonic malonate on the cleavage of *Arbacia* and *Chaetopterus* eggs.

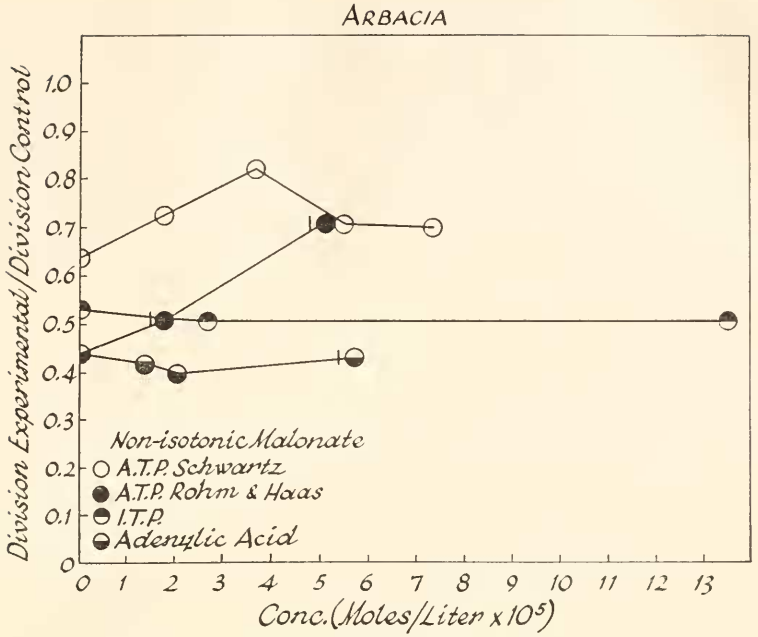

FIGURE 8. The effect of ATP, ITP and AA on non-isotonic malonate-inhibited *Arbacia* eggs. Experimental time 100 minutes.

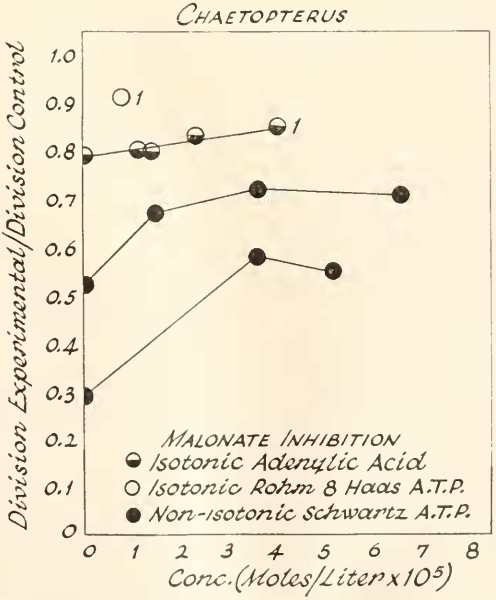

FIGURE 9. ATP and AA effect on isotonic and non-isotonic malonate-inhibited *Chaetopterus* eggs. The number indicates same experiment and same batch of eggs. Experimental time 100 minutes.

Azide, in addition to inhibiting cytochrome oxidase, is known to inhibit transphosphorylation and ATPase activity (Meyerhof, 1945). The observation that ATP was unable to reverse the azide inhibition of cleavage in *Arbacia* eggs suggested testing DNP, a substance believed to dissociate phosphorylation from oxidation (Loomis and Lipman, 1948). Figure 5 presents the data of the effect of ATP on DNP-inhibited *Arbacia* eggs. It will be noted that ATP was able partially to reverse the division inhibition. Figure 6 shows the recovery of *Arbacia* eggs after DNP inhibition. During the recovery period ATP and hydrolyzed ATP were added. The experiment shows that ATP stimulated cleavage after the removal of DNP.

Malonate inhibition of Arbacia and Chaetopterus eggs

The experimental findings obtained with malonate as the cell division inhibitor are presented in Figure 7. Figures 8 and 9 present the effect of added ATP, ITP, and AA on the malonate-inhibited *Arbacia* and *Chaetopterus* eggs, respectively. Figure 10 presents the effect of fumarate and succinate on malonate-inhibited eggs.

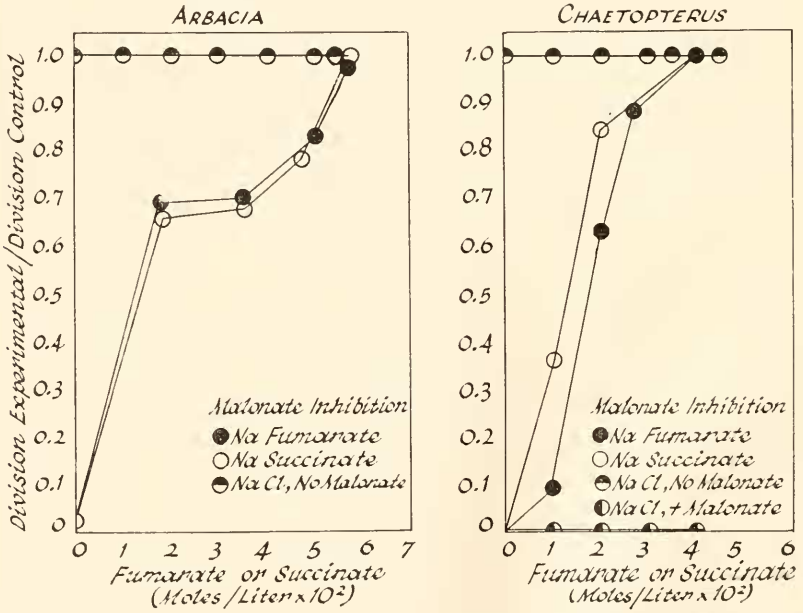

FIGURE 10. The complete reversal of malonate-inhibited *Arbacia* and *Chaetopterus* eggs by succinate and fumarate using isotonic 10^{-1} *M* (*Arbacia*) and 6×10^{-2} *M* isotonic malonate (*Chaetopterus*).

It is seen that malonate completely inhibits cleavage of both *Arbacia* and *Chaetopterus* eggs in isotonic solution. It is noted further that *Arbacia* and *Chaetopterus* eggs are 50 per cent protected against malonate by hypertonicity. At the concentrations tested, succinate and fumarate completely, and ATP incompletely, reverse the malonate inhibition. Complete reversal of the malonate

inhibition was not obtained by added ATP at the concentrations which completely reversed division inhibition due to reduced oxygen tension.

Discussion and Conclusions

Sea urchin eggs fail to divide at low oxygen tensions. From this observation Loeb (1895) and Warburg (1914) postulated that extra energy, dependent upon oxygen, was necessary for division of these eggs. Thus, if oxidative energy is involved in cell division of *Arbacia* eggs, then a decrease in the oxygen tension could inhibit division in at least two ways:

(1) Decrease in hydrogen transport, not associated with phosphorylation.
(2) Decrease in $\sim$ P formation.

An experiment performed by Korr (1939) suggests that a decrease in hydrogen transport *per se* is not primarily involved in the inhibition of *Arbacia* egg division. He added cyanide to respiring *Arbacia* eggs which resulted in a decrease in the oxygen consumption and an inhibition of division. The respiration was restored to normal by addition of methylene blue but division remained inhibited. Methylene blue is known to by-pass the dehydrogenase-cytochrome hydrogen transport system which has been shown to generate $\sim$ P (Lehninger, 1949). Consequently, these results have been interpreted as indicating that division fails because of insufficient $\sim$ P formation.

Whether reduced oxygen tension is able to decrease $\sim$ P generation and subsequently inhibit cell division depends upon the relative amount of $\sim$ P formed aerobically and anaerobically by the cell. For example, frog eggs show a very high rate of glycolysis (Barth and Jaeger, 1947). Reduction of the oxygen consumption of these eggs by cyanide or reduced oxygen tension has no effect upon division or the concentration of easily-hydrolyzed phosphate (interpreted as $\sim$ P). Addition of azide to the respiring frog eggs not only reduces the oxygen consumption and the concentration of the easily-hydrolyzable phosphate but also inhibits division. Thus, reducing the oxygen consumption of frog eggs has no effect on division; whereas, a reduction of the concentration of easily-hydrolyzable phosphate inhibits their division.

Unlike frog eggs, division of *Arbacia* eggs is inhibited when the oxygen tension is reduced, and it is not known whether the $\sim$ P concentration of the egg-cell is reduced under these conditions. It is apparent, however, from results here presented that intact ATP is able to support division of *Arbacia* eggs when their division has been inhibited by low oxygen tension. These results are indicative that division failed because of insufficient ATP and/or substances or systems dependent upon ATP. There is a linear relation between oxygen tension and division in the range where oxygen tension influences division (Clowes and Krahl, 1939). Hence, if division is dependent upon $\sim$ P, it might be expected that the amount of ATP added as $\sim$ P would be at least equivalent to that which would have been formed had the oxygen consumption remained undisturbed. The following simple calculation shows that the micromoles of $\sim$ P added as ATP is approximately ten times that calculated to be required. This calculation constitutes no proof that the ATP added was used as an energy source necessary for division. Further experiments are in progress to evaluate this point.

Normal dividing and respiring *Arbacia* eggs consume 2.4 $mm.^3$ oxygen per 10 $mm.^3$ egg hours (Hutchens *et al.*, 1942). Because 46,500 eggs occupy a volume of 10 $mm.^3$ (Harvey, 1932), the oxygen consumed per egg hour is 5.2×10^{-5} $mm.^3$. Division is normal and 50 per cent inhibited when the oxygen consumption of the *Arbacia* egg is reduced to 80 per cent and 50 per cent, respectively (Clowes and Krahl, 1939). Thus, 1.42×10^{-6} microatoms of oxygen per egg hour is the difference between the amount of oxygen consumed by normal and 50 per cent division-inhibited eggs. This oxygen consumption difference may be converted to micromoles of $\sim$ P on the assumption that three micromoles of $\sim$ P are produced per microatom of oxygen consumed (Ochoa, 1943). The result is 4.26×10^{-6} micromoles of $\sim$ P per egg hour.

The amount of ATP added to 930 eggs was 4.1×10^{-5} mM, or 2.05×10^{-5} mM of ATP per egg hour for the two-hour experimental period. ATP contains two $\sim$ P bonds (Lohman, 1938). Therefore, the amount of $\sim$ P added is 4.4×10^{-5} micromoles per egg hour as compared with 4.26×10^{-6} micromoles $\sim$ P per egg hour calculated from the oxygen consumption difference. These calculations indicate that the micromoles of $\sim$ P added as ATP are approximately ten times the amount calculated to be required.

The calculation presented is at least consistent with the concept that ATP acts to supply $\sim$ P to anoxia-inhibited eggs. However, that something in addition to ATP is necessary for division is indicated by the experiments in which ATP was added to malonate- and DNP-inhibited eggs. In both these instances, added ATP stimulated the inhibited eggs, but in no instance was the division rate re-established to normal. Greater reversal might have been obtained had higher concentrations of ATP been used. However, Runnström and Kriszat (1951) found a maximum of 40 per cent reversal when 100 times the concentration of ATP was added to DNP-inhibited sea urchin eggs.

It has been suggested (Runnström and Gustafson, 1951) that ATP could affect cell division by influencing cytoplasmic viscosity, similar to the effect of ATP on actomyosin threads and solutions. It is well known (Heilbrunn, 1943) that at the time of division there is a marked decrease in protoplasmic viscosity, and Runnström and Kriszat (1950) have shown that ATP added to unfertilized eggs decreases the cellular cytoplasmic viscosity. To test the possibility that added ATP might function in cell division as a substrate for an actomyosin-like protein, ITP, Pyro. phosphate (Pyro. phos.), and ATP were added to division inhibited eggs. These reagents have been shown to contract actomyosin threads and to decrease the viscosity of actomyosin solutions (Biro and Straub, 1949). As previously noted, of these reagents only ATP was effective in promoting division of the inhibited eggs. These results may indicate: (1) that ITP and Pyro. phos. were not accessible to the division mechanism; (2) that the egg actomyosin-like protein does not react with these substances; or (3) that the function in division of added ATP is not to support an actomyosin-like reaction. Further experiments are in progress to evaluate these points.

To summarize the results presented, the action of an inhibitor which interferes with hydrogen transport (cyanide and low oxygen tension) can be completely reversed by added ATP, provided the inhibition is not greater than 50 per cent. If either ATP utilization or the generation of metabolic intermediates are inter-

fered with (azide and DNP), ATP alone cannot make up the deficit. The fact that malonate is able completely to inhibit division of *Arbacia* and *Chaetopterus* eggs, and succinate and fumarate are able completely to reverse this inhibition, is strong evidence that division is intimately dependent upon a functioning Krebs cycle. Further, because ATP was unable to reverse completely the division inhibition due to the action of malonate, the function of the Krebs cycle in division may not be completely accounted for on the basis of $\sim$ P generation.

Summary

Division of *Arbacia* eggs was inhibited by low oxygen tension, cyanide, azide, dinitrophenol (DNP) and malonate. Various high and low energy phosphate compounds, and succinate and fumarate were added to the inhibited eggs. The results were extended to malonate-inhibited *Chaetopterus* eggs and the effects of succinate and fumarate were noted.

1. Addition of 10^{-5} to 10^{-4} *M* ATP completely reversed *Arbacia* egg division inhibition produced by cyanide and low oxygen tension. ATP incompletely reversed inhibitions caused by DNP and malonate. The maximum division stimulations were: with low oxygen tension and cyanide, division was increased from 50 per cent to 100 per cent of the control value; with 1.6×10^{-5} *M* DNP, division was increased from 23 to 51 per cent; with 7.5×10^{-2} *M* malonate, division was increased from 44 per cent to 71 per cent. With *Chaetopterus* eggs, the maximum stimulation obtained in the presence of 0.1 *M* malonate was from 28 per cent to 58 per cent of the control value.

2. Adenylic acid, hydrolyzed ATP, inorganic phosphate, and the $\sim$ P compounds, inorganic triphosphate and pyrophosphate, did not stimulate division of *Arbacia* eggs when inhibited by any of the above agents.

3. Division of *Arbacia* and *Chaetopterus* eggs was completely inhibited by 10^{-1} to 10^{-2} *M* malonate. The malonate effect was completely reversed, in both egg types, by the addition of 5.7×10^{-2} *M* succinate or fumarate.

4. Azide inhibition was not relieved by added ATP or any of the other phosphorylated compounds tested.

5. These results suggest, in *Arbacia* and *Chaetopterus* eggs, that division is intimately related to a functioning Krebs cycle, the function of which cannot be completely accounted for on the basis of $\sim$ P production.

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